Cosmetic composition comprising at least one substance promoting the formation of connexin, use and method of cosmetic treatment

The invention relates essentially to a cosmetic composition comprising at least one substance which promotes the intercellular communication of skin cells, particularly keratinocytes, fibroblasts and skin preadipocytes, and especially the formation of connexin, to its use and to a method of cosmetic treatment.

The search for means of combating human ageing currently receives the attention of a large number of researchers. This is particularly the case in the field of cosmetics, where attempts are made to combat, or at least slow down, the appearance of the esthetic and physiological effects of skin ageing, such as wrinkles, loss of elasticity of the skin, loss of color, etc.

Ageing of the human species is characterized by numerous disorders affecting the living tissues. The signs of this natural process are easily detectable on an organ such as the skin (Montagna, W. & Carlisle, K. (1990). Structural changes in ageing skin. British Journal of Dermatology, 122(35), 61-70). Skin ageing is a combination of two phenomena, namely an intrinsic (genetic) cellular process associated with so-called extrinsic ageing, which groups together environmental aggressions (Grove, G.L. (1989). Physiologic changes in older skin. Clinics in geriatric medicine, 5(1), 115-125). The effects of senescence on skin tissue are visible mainly in the formation of wrinkles. These reflect the substantial changes which take place in the dermis and the epidermis. Tissue homeostasis, which is important for the skin, is modified in the course of ageing. The gap junctions participate in the regulation of cell homeostasis, so their role in maintaining the physiological equilibrium of the skin is important.

The inventors have demonstrated an ageing effect on the functional capacity of cells to communicate with one another, especially via the gap junctions (GJ), and also an ageing effect on the amount of connexin 43 present in the cells. In fact, they have demonstrated that the functional capacity of cells to communicate with one another, especially via the gap junctions (GJ), decreases with age. They have also demonstrated that the amount of connexin 43 decreases with age. The inventors have therefore shown the value of using substances which promote intercellular communication, especially via the GJ, for combating the signs of skin ageing and particularly for slowing down their appearance. This represents a new means of combating skin ageing, particularly wrinkles and the loss of elasticity of the skin.

The gap junctions (GJ) are transmembrane protein structures which allow small molecules (<1000 Da) to pass between two cells (Watt, F.M. (1991). Intercellular communication via gap junctions. In L.A. Goldsmith (Eds.), Physiology, biochemistry, molecular biology of the skin (pp. 857-859). New York, Oxford: Oxford University Press). These hexameric structures are called connexons, which are themselves formed from connexins.

When an intercellular contact is established, the connexons of one cell align end-to-end with those of the adjacent cell to form a junctional channel (Goodenough, D.A., Golier, J.A. & Paul, D.L. (1996). Connexins, connexons and cellular communication. Annual Review of Biochemistry, 65, 475-502). These gap junctions (GJ) make it possible to maintain the cell and tissue homeostasis. Connexins form part of a family of proteins each having tissue specificities. Connexin 43 is the majority protein in keratinocytes (Salomon, D., Saurat, J.-H. & P.M. (1988). Cell-to-cell communication within intact human skin. Journal of Clinical Investigation, 82, 248-254). In skin the GJ are present in all the layers of the epidermis except the stratum corneum.

Furthermore, it is known that molecules which affect the way cells function can do this either by entering the cells or by not entering the cells but binding to receptors present on the plasma membrane, thus triggering a series of reactions which culminate in the release of small molecules, called "second messengers", in the cytoplasm. These small second messengers, for example cAMP (cyclic adenosine 3',5'-monophosphate), circulate from one cell to the next via the GJ, thereby transmitting the information from cell to cell. The same applies to small molecules which enter the cells and are capable of circulating from one cell to the next via the GJ. Thus, by increasing the intercellular communication, especially via the GJ, the inventors have developed a novel means of promoting and/or increasing the activity of a cosmetic agent.

The inventors have also shown that, surprisingly, the use of boldine or, independently, the use of lipid extracts of the alga Skeletonema costatum makes it possible to restore intercellular communication, especially via the GJ, and also to increase the level of connexin 43 present in skin cells, especially keratinocytes, fibroblasts and preadipocytes.

Thus the main object of the present invention is to solve the new technical problem which consists in the provision of a novel solution for improving the efficacy of cosmetic compositions and particularly for combating, or at least

slowing down, the appearance of the esthetic and physiological effects of skin ageing, such as wrinkles, loss of elasticity of the skin, loss of color, etc.

According to the present invention, this technical problem has been solved for the first time, in a surprising and non-obvious manner, by the discovery that a substance which promotes the intercellular communication of keratinocytes, fibroblasts and skin preadipocytes makes it possible to provide a cosmetic composition which has improved properties, particularly for combating, or at least slowing down, the appearance of the esthetic and physiological effects of skin ageing, such as wrinkles, loss of elasticity of the skin, loss of color, etc.

Furthermore, the present invention has afforded the discovery, again in an unexpected and non-obvious manner, that a substance which promotes the formation of connexin, particularly connexin 43, makes it possible to prepare a cosmetic composition of enhanced efficacy, particularly for combating, or at least slowing down, the above-mentioned appearance of the esthetic and physiological effects of skin ageing.

According to the present invention, it has also been discovered, in an unexpected and non-obvious manner, that on the one hand boldine and on the other hand, independently, a lipid extract of the alga Skeletonema, especially the alga Skeletonema costatum, and particularly a total lipid extract of said alga, promote intercellular communication via the gap junctions of skin cells, particularly keratinocytes, fibroblasts and skin preadipocytes, and thus makes it possible to prepare a cosmetic composition of enhanced efficacy, particularly for combating or slowing down the appearance of the esthetic and physiological effects of skin ageing or for combating hyperadiposis.

Thus, according to a first feature, the present invention relates to a cosmetic composition which is characterized in that it comprises, as an active ingredient, at least one substance which promotes the intercellular communication of skin cells, particularly keratinocytes, fibroblasts and skin preadipocytes.

In one particular embodiment of the invention, the composition is characterized in that said substance promotes intercellular communication via the gap junctions of skin cells, particularly keratinocytes, fibroblasts and skin preadipocytes.

In another advantageous embodiment of the invention, the composition is characterized in that said substance which promotes intercellular communication promotes the formation of connexin, particularly connexin 43.

In yet another advantageous embodiment of the invention, the above-mentioned composition is characterized in that said substance which promotes intercellular communication comprises at least one lipid extract of the alga Skeletonema, especially the alga Skeletonema costatum, and particularly a total lipid extract of said alga.

It is pointed out that the alga Skeletonema, particularly Skeletonema costatum (called SKC in the remainder of the document), is a well-known single-cell alga of the phylum Chlorophytes, the branch Chrysophycophytes, the class Diatomophyceae and the order Centrales. Diatomophyceae are very widespread in fresh, salt or brackish waters. The life of the species of this class can be planktonic or benthic. The protoplasm is enclosed in a siliceous frustule. Skeletonema costatum (SKC) is a cosmopolitan and usually marine species, which is frequently found to be associated with the phytoplanktonic efflorescences of coastal waters.

In one advantageous embodiment of the invention, this lipid extract is characterized in that it is obtained by extracting the alga Skeletonema with an alcoholic solvent selected from the group consisting of isopropanol, ethanol and methanol.

In another advantageous variant, the extraction is performed under reflux.

In another advantageous variant, the alga is frozen before being extracted with the alcoholic solvent, the freezing preferably being effected at a temperature of between about -40°C and -20°C and for a period preferably of between about 1 and 7 days.

In another advantageous variant, the frozen alga is immersed directly in the heated alcoholic solvent. The thermal shock in fact makes it possible to facilitate the decantation of the silica (originating from the skeleton of the algal cells).

In another advantageous variant, in the case of extraction with an alkalized alcohol, the above-mentioned algal extract is obtained after the following series of steps:

a) the alcoholic solvent is alkalized to a pH of between 10 and 14, preferably to a pH of 13, for example with aqueous sodium hydroxide solution or aqueous potassium hydroxide solution,
b) the insoluble materials are removed from the aqueous-alcoholic phase,
c) distilled water is added to the aqueous-alcoholic phase,
d) the solution obtained is subjected to liquid-liquid extraction with an apolar solvent immiscible with the aqueous-alcoholic phase, for example heptane,

hexane or cyclohexane,

e) the phase containing the apolar solvent is removed,

f) the aqueous-alcoholic phase recovered after removal of the phase containing the apolar solvent is acidified to a pH of between 1 and 3, preferably to a pH of 2, for example with aqueous sulfuric acid solution or aqueous hydrochloric acid solution,

g) the solution obtained after acidification is subjected to liquid-liquid extraction with an apolar solvent immiscible with the aqueous-alcoholic phase, for example heptane, hexane or cyclohexane,

h) the aqueous-alcoholic phase is removed, and

i) the phase containing the apolar solvent recovered after removal of the aqueous-alcoholic phase is evaporated to give an oil free of apolar solvent, this oil being the target extract.

The use of an alkalized and then acidified alcohol affords an extract with visual and olfactory characteristics that are acceptable in cosmetic compositions (yellow coloration and acceptable odor).

In another advantageous variant, the above-mentioned extract is obtained by extraction with supercritical CO_2.

In yet another advantageous variant, before any other extraction operation, the alga is macerated in the alcoholic solvent at room temperature, preferably for a period of between about 5 minutes and 80 minutes and particularly preferably for a period of between about 20 minutes and 40 minutes.

In yet another advantageous variant, the amount of alcoholic solvent used is between about 0.1 liter and 20 liters of solvent, preferably between about 2 liters and 10 liters of solvent, per 100 g of alga, expressed by dry weight of alga.

In yet another advantageous variant, the extraction can be performed under an inert atmosphere, preferably a nitrogen-saturated atmosphere. This makes it possible in particular to avoid pronounced oxidative degradation of the active molecules.

This lipid extract is preferably packaged under an inert gas, such as nitrogen, in order to protect the active molecules.

In yet another advantageous variant, the above-mentioned composition is characterized in that it comprises from about 0.01% to 10% and particularly from about 0.1% to 3% by weight of said lipid extract of the alga Skeletonema, especially the alga Skeletonema costatum, based on the total weight of the final

composition.

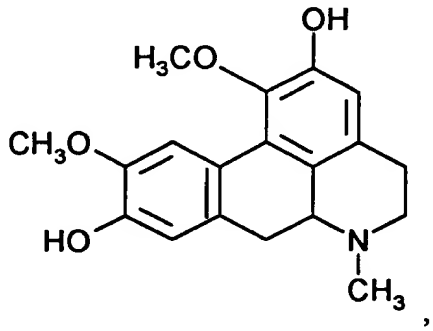
According to a second feature, the present invention further relates to the use of at least one substance which promotes the intercellular communication of keratinocytes, fibroblasts and skin preadipocytes as a cosmetic agent, optionally in the presence of a cosmetically acceptable vehicle.

In one particular embodiment of the use, the latter is characterized in that said substance promotes intercellular communication via the gap junctions of keratinocytes, fibroblasts and skin preadipocytes.

In another particular embodiment of the invention, the substance which promotes intercellular communication promotes the formation of connexin, particularly connexin 43.

In another embodiment of the invention, the substance comprises at least one lipid extract of the alga Skeletonema, especially the alga Skeletonema costatum, and particularly a total lipid extract of said alga, especially as defined above. Advantageously, this above-mentioned extract is obtained by liquid-liquid extraction between an alkalized and then acidified alcohol and an apolar solvent immiscible with the aqueous-alcoholic phase, for example heptane, hexane or cyclohexane.

In another embodiment of the invention, the substance which promotes the intercellular communication of skin cells is boldine, a product of the following formula:



the compositions of the invention which contain said boldine advantageously containing it in an amount of about 0.001 to 10% and advantageously of 0.01 to 1% by weight.

According to a third feature, the present invention also covers a method of promoting and/or increasing the activity of a cosmetic agent acting directly in the cell or via intracellular second messengers, characterized in that it comprises the application, simultaneously with or prior to that of said cosmetic agent, to the

appropriate skin areas of a person in need thereof, of an effective amount of at least one substance promoting intercellular communication, particularly a substance promoting intercellular communication as defined above.

According to a fourth feature, the present invention also covers a method of cosmetic skin anti-ageing treatment, characterized in that it comprises the application, to the appropriate skin areas of a person in need thereof, of an effective amount of at least one substance promoting intercellular communication for obtaining an anti-ageing effect on said skin areas, especially for improving the firmness and elasticity of the skin, for delaying the appearance of wrinkles or for reducing their depth, and particularly a substance promoting intercellular communication as defined above.

The Examples which follow are given purely to illustrate the invention with reference to the attached drawings, in which:

- Figure 1 shows the curve representing the modulation of intercellular communication as a function of donor age. It is obtained from keratinocytes isolated from donors of different ages by the so-called scrape-loading method. The curve is expressed as the ratio of the fluorescent surface area to the total surface area on the ordinate as a function of the donor age in years on the abscissa. "r" is the correlation coefficient, which in this case is equal to 0.76. The measurements obtained with young donors are represented by filled squares with the standard deviation and the measurements denoted by blank circles were obtained with old donors, again with the standard deviation.

This Figure should be read in conjunction with Table I (Example I).

- Likewise, Figure 2 again shows a curve representing the modulation of intercellular communication as a function of donor age, obtained by a microinjection method, except that this time the curve is expressed as the number of labeled keratinocytes on the ordinate as a function of the age expressed in years on the abscissa. r again represents the correlation coefficient, which in this case is equal to 0.91. The measurements obtained with young donors are denoted by black squares with their standard deviation and the measurements obtained with old donors are denoted by blank circles with their standard deviation.

This Figure should be read in conjunction with Table II (Example I).

- Figure 3 shows the change in the level of connexin 43 measured on keratinocytes of donors of different ages by flux cytometry and expressed as the percentage labeling on the ordinate as a function of the donor age (in years) on the

abscissa, with a correlation coefficient r, which in this case is equal to 0.82. The measurements obtained with young donors are denoted by black squares with their standard deviation and the measurements obtained with old donors are denoted by blank circles with their standard deviation.

This Figure should be read in conjunction with Table III (Example I).

- Figure 4 shows the results of modulation of the intercellular communication of normal human keratinocytes, denoted by NHK, of different donors who may or may not have been treated with a lipid extract of the alga Skeletonema costatum, abbreviated to SKC, the results being represented in the form of histograms and expressed as relative diffusion units on the ordinate, the blank bar being obtained with control NHK and the shaded bar being obtained with NHK treated in vitro with a lipid extract of the alga Skeletonema costatum in a proportion of 2.5 µg/ml/24 h.

This Figure should be read in conjunction with Table VI (Example III).

- Figure 5 shows the measurement, by the microinjection technique, of the modulation of the intercellular communication of normal human keratinocytes (abbreviated to NHK) of different donors who may or may not have been treated with a lipid extract of the alga Skeletonema costatum, abbreviated to SKC, with the number of labeled cells on the ordinate, the results with the untreated, control NHK being shown by a blank bar marked "control", and the NHK treated with a lipid extract of the alga Skeletonema costatum, or SKC, at a dose of 2.5 µg/ml/24 h being shown by a black bar marked "treated".

This Figure should be read in conjunction with Table V (Example III).

- Figure 6 shows another histogram, obtained from results measured by flux cytometry, of the modulation of the amount of connexin 43, relative to their respective controls, in donors of different ages treated with the lipid extract of the alga Skeletonema costatum, abbreviated to SKC, at a dose of 2.5 µg/ml/24 h, with the percentage increase relative to their respective controls on the ordinate and the donor age in years on the abscissa.

This Figure should be read in conjunction with Table VI (Example III).

Other advantages of the invention will become apparent from the description and Examples which follow.

Unless indicated otherwise, the proportions given in the Examples of compositions are expressed as percentages by weight.

Example I - Demonstration of the decrease in functionality of the gap junctional intercellular communication (GJIC) with age and the decrease in

the amount of connexin 43 with age

I.1- Materials and methods

I.1.1- Culture of normal human keratinocytes

The cultures of normal human keratinocytes (NHK) are prepared from skin samples.

The sample is first rinsed 4 times in PBS (Phosphate Buffered Saline - Sigma) (50 ml tubes). It is then decontaminated by successive immersion in two baths of 70% ethanol for 30 seconds. When the sample has been decontaminated, it is placed in a Petri dish containing PBS. 1 mm wide strips are then cut out, care being taken to remove as much fatty tissue and dermis as possible. The strips are placed immediately in a Petri dish containing PBS.

For recovery of the keratinocytes from the epidermis, the strips are placed for 4 h at 37°C in a 0.25% solution of trypsin in PBS.

The dermis is then separated from the epidermis by scraping the strips with a scalpel, the epidermal cells obtained being suspended in a tube containing DMEM (Dulbecco Modified Eagle's Medium - Gibco) + 10% FCS (Fetal Calf Serum - Eurobio). After homogenization of the suspension, the surface portion consisting of stratum corneum cells is removed and the remaining suspension is filtered on a sieve.

The filtered portion is centrifuged for 5 minutes at 176 g. The residue is taken up with NHK-D medium (DMEM + 10% FCS + 0.4 µg/ml hydrocortisone + 10 ng/ml EGF + 10^{-9} M cholera toxin). The cells are counted and then inoculated at a rate of 15 x 10^6 cells/flask.

After 24 h of culture, the medium is changed, the cells are rinsed with PBS and K-SFM proliferation medium (Gibco) is used for the remainder of the culture.

The keratinocytes are subcultured in totally conventional manner, but they have to be subcultured at 60-70% of confluence in order to retain their capacity to proliferate. Thus, when the cells are at 60-70% of confluence, the maintenance medium is removed and the cellular mat is rinsed with PBS. The cells are placed in 3 ml of trypsin/EDTA solution; then, when the cells detach, the trypsin is inhibited with a medium containing 10% of FCS (Eurobio). The cell suspension is homogenized, recovered and then centrifuged at 20 g for 5 minutes at room temperature. The resulting residue is taken up with medium only. For

maintenance, inoculation is carried out as before at a rate of 10^6 cells/75 cm^2 in ventilated flasks stored under the conditions mentioned above. Confluence is obtained after about ten days and the cells can be amplified over 6 to 7 passes.

In the case where it is desired to test the activity of a substance on these keratinocytes, the tests will be performed using the culture medium containing keratinocytes at the moment of confluence described above.

I.1.2- Scrape-loading

This technique was developed in 1987 by Trosko's group (El-Fouly, M.H., Trosko, J.E. & Chang, C.-C. (1987). Scrape loading and dye transfer - a rapid and simple technique to study gap junctional intercellular communication. Experimental Cell Research, 168, 422-430). It is based on the use of the dye Lucifer Yellow (Sigma) of molecular weight (MW) 457.2. This molecule (an aminophthalimide) is highly fluorescent and only diffuses through the gap junctions. It does not pass through the plasma membrane of intact cells. The cellular layer is injured with a scalpel, allowing the dye access to the gap junctions.

The cells at confluence are rinsed with PBS (Sigma) before the dye is added. 2 ml of 0.05% Lucifer Yellow (Sigma) diluted in PBS are deposited on the cells and the scrape is carried out at room temperature. Six scrapes are carried out in 60 mm dishes. The cells are left in contact with the dye for 5 minutes, the latter then being withdrawn and recovered. The cells are then rinsed with PBS to remove the excess fluorescence. They are fixed with 3% paraformaldehyde (PFA) for 10 minutes. The cells are rinsed again with PBS, 2 ml of PBS being left in the dishes after rinsing.

The dishes are then observed with an inverted fluorescence microscope fitted with an image acquisition camera. Six or eight photographs are taken for each dish and then analyzed with an image analyzing software (Perfectimage, Iconix). The fluorescence corresponding to the surface area occupied by the Lucifer Yellow in the cellular layers is then quantified. This is done by placing a rectangle of predefined size in the zone to be studied. The computer calculates the surface area ratio: fluorescent surface area/total surface area. The higher the ratio, the greater is the capacity of the cells to communicate with one another.

I.1.3- Microinjection

The cells are prepared in a manner similar to that used by the scrape-loading technique.

The precision of this technique allows the Lucifer Yellow (10% in 0.33 M LiCl) to be injected directly into a cell solely by means of a capillary connected to a micromanipulator (Leitz). The injection is controlled by an automatic injector (Tranjector 5246, Eppendorf) and lasts 1 second with an intensity of 10,335 HPa. The injections are performed under an inverted microscope (20X) in the phase contrast mode (Leica microscope, Fluovert FU). About twenty injections are performed in a 60 mm Petri dish.

After the cells have been fixed with 3% paraformaldehyde, they are rinsed with PBS. This is followed by observation and counting of the number of labeled cells.

I.1.4- Flux cytometry

When confluence is reached, the culture medium is removed and the cells are rinsed twice with PBS. They are then trypsinized with a mixture of 0.1% trypsin and 0.02% EDTA. Inhibition of the trypsin is effected with culture medium supplemented with 10% of FCS. The number of cells is counted so that it can be adjusted to 10^6 cells per tube. The cell suspension is centrifuged at 176 g for 5 minutes and the residue is taken up with 100 ml of PBS. The cells are fixed with 3% PFA for 25 minutes at 4°C. To remove the PFA, the cells are centrifuged again at 176 g for 5 minutes and rinsed twice in PBS.

The buffer in which the antibodies are prepared is composed of PBS, 1% of FCS and 0.2% saponin (Sigma), a detergent which induces microporation of the membrane but does not damage the cell. This detergent is used to keep both the intracytoplasmic and the transmembrane connexin 43 intact and quantify it (personal communication, R. Mouawad, Hôpital Salpétrière - Paris).

The primary antibody used is anti-connexin 43 (Zymed, monoclonal antibody created in the mouse). The concentration of the primary antibody must be 1.3 µg/ml (1/750). Incubation is carried out for 45 minutes at 4°C. To remove the excess antibody, the cells are centrifuged and rinsed with the buffer used to prepare the antibodies (PBS, saponin and FCS). They are then brought into contact with the secondary antibody, a fluorescein-coupled anti-mouse antibody, at a dilution of 1/50 (Jackson Immunotech). After incubation with the second antibody, the cells

are centrifuged and rinsed and then taken up with 1 ml of buffer (PBS, saponin, FCS).

The fluorescence intensity in the NHK treated as described above and in NHK which have not been brought into contact with the fluorescein-coupled anti-mouse secondary antibody is measured with a flux cytometer (Epics profile II from COULTRONIC) and the measurements are processed and compared by a software (Phoenix flow system soft from COULTRONIC), which provides a value measured in an arbitrary unit given by the machine. This is called the level of labeling of the connexin 43 in the NHK, which reflects the amount of connexin 43 per NHK (averaged).

I.2- Results

I.2.1- Evaluation of the functionality of gap junctional intercellular communication (GJIC) as a function of donor age

I.2.1.1- Scrape-loading (Figure 1)

The study was conducted on 9 donors aged between 40 and 75 years.

Table I

Modulation of intercellular communication as a function of donor age

Donor age (years)	Fluorescent surface area/total surface area (mean)	Standard deviation
40	0.41	0.06
41	0.40	0.05
43	0.60	0.07
47	0.33	0.14
48	0.10	0.08
67	0.31	0.06
68	0.33	0.08
70	0.25	0.05
75	0.21	0.04

From the results shown in Table I and Figure 1 (attached), it can be seen that intercellular communication decreases with donor age, the capacity of the keratinocytes to communicate apparently being inversely proportional to donor age.

The correlation coefficient is 0.76.

I.2.1.2- Microinjections (Figure 2)

The study was conducted on the same donors as for the scrape-loading, with the exception of the 43-year-old donor.

Table II

Modulation of intercellular communication as a function of donor age

Donor age (years)	Number of labeled cells (mean)	Standard deviation
40	15.1	3.6
41	16.9	5.4
47	11.8	3.2
48	10.1	2.2
67	8.6	2.7
68	8.7	2.6
70	5.9	2.4
75	6.8	1.7

From the results shown in Table II and Figure 2 (attached), we see that GJIC is inversely proportional to age; however, with this technique, which is more precise than scrape-loading, the correlation coefficient is 0.91.

The two techniques, scrape-loading and microinjections, give results which show the same trend: the functionality of GJIC decreases very significantly with age.

I.2.2- Evaluation of the effect of age on the amount of connexin 43 (Figure 3)

Flux cytometry

The study was conducted on the same donors as for the scrape-loading, with the exception of the 43-year-old donor and the 75-year-old donor.

Table III

Level of labeling of connexin 43 in NHK, measured on keratinocytes of donors of different ages

Donor age (years)	Level of labeling of connexin 43 in NHK (mean)	Standard deviation
40	55.1	4.7
41	54.5	7.5
47	42.1	2.5
48	28.9	1.8
67	19.1	0.8
68	27.1	7.1
70	25.6	2.0

It is seen from Table III and Figure 3 (attached) that the level of connexin 43 decreases with age, the amount of connexin 43 apparently being inversely proportional to donor age.

The correlation coefficient is 0.82.

These results show that there is an ageing effect on the functional capacity of cells to communicate with one another, especially via the gap junctions (GJ), and an ageing effect on the amount of connexin 43 present in the cells. In fact, they show that the functional capacity of cells to communicate with one another, especially via the gap junctions (GJ), decreases with age. They also show that the amount of connexin 43 decreases with age.

The inventors have therefore shown the value of using substances which promote intercellular communication, especially via the GJ, for combating the signs of skin ageing and particularly for slowing down their appearance. They have therefore developed a novel means of combating skin ageing, particularly wrinkles and the loss of elasticity of the skin.

The inventors have therefore also shown the value of using substances which promote intercellular communication, especially via the GJ, for promoting and/or increasing the activity of a cosmetic agent. They have therefore developed a novel means of promoting and/or increasing the activity of a cosmetic agent.

Example II - Preparation of lipid extracts of the alga Skeletonema costatum

II.1- Extraction with isopropanol by a first method

Preferably, the whole extraction will be performed under an inert atmosphere (nitrogen saturation) in order to avoid pronounced degradation of the active molecules.

250 kg of biomass (Skeletonema costatum) are used in this preparation.

The algae, which have been frozen to -20°C, are immersed in isopropanol (IPA) refluxing at 80-83°C, with agitation. The thermal shock makes it possible to facilitate the decantation of the silica (originating from the skeleton of the algal cells).

The amount of solvent used is 10 liters of IPA per liter of water present in the biomass. In this preparation, for a proportion of dry matter of 30%, the 250 kg of biomass represent 75 kg of dry matter and 175 kg of water. The amount of IPA used here is 1925 kg.

The whole (biomass + IPA) is refluxed for half an hour at about 80°C, with agitation, before being cooled to about 50°C.

After the reaction mixture has been cooled to about 50°C, the extract is transferred to a GUEDU filter in order to separate the extracted biomass from the IPA lipid extract.

The lipid extract is concentrated in a batch reactor (concentration factor = 71.5).

The yield of crude oil in this first step is 28%, based on dry weight.

To start the second step, the lipid extract is taken up with cold IPA at a rate of 10 kg of solvent per kg of oil. Agitation is continued for 20 minutes. The liquor is then filtered (enabling the residual sticky sludge to be removed).

The decolorization and deodorization treatment is carried out in two batches in an 80-liter Schott reactor and takes 30 minutes at room temperature. 0.94 kg of zeolite (ABSENT 2000 supplied by UOP) and 1.6 kg of active charcoal (CXV supplied by CECA) are added, the charcoal-to-zeolite ratio being 1.7.

The zeolite and charcoal are then filtered off on paper.

The yield of this second step is 37%, based on dry weight.

Thus the overall yield of oil for the process as a whole is 10%, based on dry weight of biomass.

Antioxidants (DL-α-tocopherol at a final concentration of 0.05% by weight

and ascorbyl palmitate at a final concentration of 0.05% by weight) are incorporated by way of a stock solution in IPA.

The filtrate and antioxidants are then concentrated batchwise to give a brown-colored oil.

This lipid extract (in the form of an oil) is packaged under an inert gas such as nitrogen.

This oil will be referred to below as SKC lipid extract E1.

II.2- Extraction with ethanol by a second method

The extraction begins with the dispersion of 49.8 kg of frozen vegetable biomass (29% of dry matter) in 539 kg of 96% ethanol alkalized with 9 kg of 30.5% aqueous sodium hydroxide solution. After maceration for 30 minutes at 40°C under reflux and under a nitrogen atmosphere, the whole is cooled to 18°C.

The insoluble materials are then filtered off under nitrogen and removed.

151 kg of distilled water are added to the 573.9 kg of filtrate. The whole is agitated slowly for 10 minutes before being added to 162 kg of heptane. The heptane epiphase of the liquid-liquid partition is removed. It is pointed out that the hypophase contains the fatty acids in the form of salts. The operation is repeated two more times.

The 756 kg of solution constituting the above-mentioned hypophase are acidified by the addition of 2.8 kg of sulfuric acid to give a pH of 2.2. The whole is agitated for 10 minutes under nitrogen before being added to 158 kg of heptane. The free fatty acids are extracted from the 147 kg constituting the first heptane epiphase of this new liquid-liquid partition. The operation is repeated five more times to recover a total of 697 kg of heptane phase. This phase is evaporated to dryness on a rotary evaporator and then by molecular distillation to give the active extract in an amount representing 1.1 kg of oil.

The oil produced is a dark yellow-colored homogeneous liquid.

This oil will be referred to below as SKC lipid extract E2.

Example III - Evaluation of the effect of a lipid extract of the alga SKC on gap junctional intercellular communication (GJIC) and the level of connexin 43 in NHK of old donors

The lipid extract of the alga SKC used in this Example is SKC lipid extract E2 obtained by the ethanol extraction method described above.

The studies described below were also conducted with SKC lipid extract E1 (results not shown), the results being comparable to those described below and obtained with SKC lipid extract E2.

The studies are conducted on normal human keratinocytes (NHK) obtained according to the protocol described in Example I (I.1.1 - Culture of normal human keratinocytes). In contrast to the controls, the NHK treated with SKC lipid extract E1 or E2 (at the moment of confluence of the culture) are brought into contact with an amount "x" of said extract, expressed in µg per milliliter of culture medium per 24 hours (x µg/ml/24 h), before being evaluated by the techniques of scrape-loading, microinjections or flux cytometry according to the protocols described in Example I (I.1.2, I.1.3 and I.1.4 respectively).

• Scrape-loading (Figure 4)

The study was conducted on normal human keratinocytes (NHK) of 4 donors aged between 60 and 79 years.

Table IV

Modulation of the intercellular communication of NHK of different donors treated with SKC lipid extracts (E2), measured by the scrape-loading method

Donor		Treatment and dose			
		SKC lipid extracts (E2)			Control
Age	Data	1.25 µg/ml	2.5 µg/ml	5 µg/ml	0
63 years	R (mean)	0.55	0.53	0.50	0.38
	Stand. dev.	0.05	0.07	0.04	0.07
79 years	R (mean)	0.53	0.54	0.59	0.42
	Stand. dev.	0.06	0.06	0.11	0.07
60 years	R (mean)	0.51	0.54	0.57	0.38
	Stand. dev.	0.07	0.06	0.05	0.06
73 years	R (mean)	0.61	0.56	0.65	0.49
	Stand. dev.	0.05	0.07	0.07	0.04

"R" is the ratio of the fluorescent surface area to the total surface area.

The results shown in Table IV and Figure 4 (attached) demonstrate that the SKC lipid extracts induce an increase in GJIC in old donors ($p < 0.0001$ - Student

test).

The SKC lipid extracts therefore restore cellular communication to approximately the same level as in NHK of young donors.

• Microinjections (Figure 5)

The study was conducted on 12 donors aged between 49 and 79 years.

Table V

Modulation of the intercellular communication of NHK of different donors treated with SKC lipid extracts (E2)

Donor		Treatment and dose	
		SKC lipid extract (E2)	Control
Age	Data	2.5 µg/ml	0
49 years	Number of labeled cells (mean)	15.9	7.7
	Standard deviation	1.5	2.0
52 years	Number of labeled cells (mean)	21.4	10.9
	Standard deviation	2.6	2.1
55 years	Number of labeled cells (mean)	19.0	8.7
	Standard deviation	2.2	1.2
55 years	Number of labeled cells (mean)	13.9	8.6
	Standard deviation	2.8	2.1
59 years	Number of labeled cells (mean)	13.1	5.3
	Standard deviation	1.7	0.9
59 years	Number of labeled cells (mean)	23.4	11.0
	Standard deviation	4.3	1.9
60 years	Number of labeled cells (mean)	12.4	8.4
	Standard deviation	1.7	1.9
61 years	Number of labeled cells (mean)	13.8	8.1
	Standard deviation	1.4	1.8
62 years	Number of labeled cells (mean)	19.6	10.0
	Standard deviation	3.9	2.9
65 years	Number of labeled cells (mean)	23.9	10.9
	Standard deviation	3.5	2.7
71 years	Number of labeled cells (mean)	9.5	4.1
	Standard deviation	1.3	1.2
79 years	Number of labeled cells (mean)	13.9	8.3
	Standard deviation	1.9	1.8

The number of labeled cells is seen to increase by a factor of 2 ($p < 0.0001$ - Student test). The SKC lipid extracts therefore induce a restoration of the GJIC of cells of old donors to approximately the level of the GJIC of cells of young donors.

• Flux cytometry (Figure 6)

The study was conducted on 6 donors aged between 49 and 60 years.

Table VI

Modulation of the amount of connexin 43 after treatment with SKC lipid extract (E2) at 2.5 µg/ml/24 h, measured by flux cytometry

Donor age (years)	Percentage increase in the amount of connexin 43 relative to the respective controls (%)
49	31.5
52	25.5
55	30.8
59	11.5
59	19.0
60	35.2

A mean increase of 25.6% in the labeling of the treated cells relative to the control cells is observed.

The SKC lipid extracts induce an increase in the amount of connexin 43.

The results obtained with the different means of evaluating the effect of lipid extracts of the alga Skeletonema costatum on intercellular communication, especially via the GJ, and on the level of connexin 43, carried out on NHK of old donors, show that the use of lipid extracts of the alga Skeletonema costatum makes it possible to restore intercellular communication, especially via the GJ, and also to increase the level of connexin 43 present in the NHK.

These results show the value of using lipid extracts of the alga Skeletonema costatum in cosmetic compositions for combating the signs of skin ageing and particularly for slowing down their appearance. The inventors have therefore developed a novel means of combating skin ageing, particularly wrinkles and the loss of elasticity of the skin.

Furthermore, the results obtained above show the value of using lipid extracts of the alga Skeletonema costatum in cosmetic compositions for promoting and/or increasing the activity of other cosmetic agents which act directly in the cell or via intracellular second messengers, and which may or may not be present in the same cosmetic composition as the SKC lipid extracts.

Example IV - Evaluation of the effect of boldine by the scrape-loading test

The study was conducted on normal human keratinocytes (NHK), the concentrations used being 12.5, 25, 50, 100 and 200 mM. The incubations take 24 h in the culture medium. The Table below summarizes the measured values of the surface area of diffusion.

Table VII

Concentration of boldine (mM)	control	15.5	25	50	100	200
Mean surface area of diffusion	0.46	0.47	0.51	0.574	0.512	0.39
Standard deviation	0.07	0.06	0.04	0.03	0.66	0.08

As can be seen in Table VII, the dose which seems to be the most effective for increasing the functionality of GJIC is 50 mM/24 h. Statistical analysis of the data shows that only this concentration affords a really significant difference in the increase in GJIC relative to the untreated NHK. The results in Table VII suggest that boldine has a dose-related effect on GJIC.

Example V - Anti-ageing day cream for the face

Glyceryl stearate + PEG-100 stearate	6.00 %
Squalane	3.00 %
Hydrogenated polyisobutene	3.00 %
Glycerol tricaprylate/caprate	3.00 %
Glycerol	2.00 %
Octyl methoxycinnamate	2.00 %
Beeswax	1.50 %
Cetostearyl octanoate	1.50 %
Cetyl alcohol	1.00 %
Stearyl alcohol	1.00 %
Dimethicone	1.00 %
SKC lipid extract E2	1.00 %
Xanthan gum	0.20 %
Carbomer	0.15 %
Neutralizing agent	qs
Preservatives	qs
Perfume, colors	qs
Water	qsp 100.00 %

Example VI - Anti-wrinkle emulsion-gel for the face

Glycerol	5.00 %
Caprylic/capric/succinic triglycerides	3.00 %
SKC lipid extract E2	2.00 %
Octyl methoxycinnamate	1.00 %
Acrylates/C10-30 alkyl acrylate crosspolymer	0.50 %
Wheat protein hydrolyzate	0.50 %
Dimethicone copolyol	0.50 %
Neutralizing agent	qs
Preservatives	qs
Perfume, colors	qs
Water	qsp 100.00 %

Example VII - Firming emulsion for the body

Octyl palmitate	7.00 %
Glycerol tricaprylate/caprate	3.00 %
Octyl octanoate	2.00 %
Phenyltrimethicone	2.00 %
Glycerol	2.00 %
Stearic acid	1.00 %
Sorbitan stearate, 20 EO	0.90 %
Cetyl alcohol	0.50 %
Stearyl alcohol	0.50 %
SKC lipid extract E2	0.50 %
Carbomer	0.40 %
Xanthan gum	0.20 %
Sorbitan stearate	0.10 %
Neutralizing agent	qs
Preservatives	qs
Perfume, colors	qs
Water	qsp 100.00 %

Example VIII - Anti-wrinkle emulsion-gel for the face

Glycerol	5.00%
Caprylic/capric/succinic triglycerides	3.00%
Octyl methoxycinnamate	1.00%
Acrylates/C10-30 alkyl acrylate crosspolymer	0.50%
Wheat protein hydrolyzate	0.50%
Dimethicone copolyol	0.50%
Boldine	0.01%
Neutralizing agent	qs
Preservatives	qs
Perfume, colors	qs
Water	qsp 100.00%